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13. ABSTRACT (Maximum 200 words)

The principal outcome of this research has been to integrate fundamental enzymatic, genetic and physiological studies with mathematical analysis to better define the factors controlling the rate of biodegradation of selected model compounds representative of military toxic wastes. The worked focuses on further developing the biodegradation potential of the broad-spectrum organophosphorus hydrolase (OPH) enzyme when used alone, in enzyme-based complexes, as well as in microbial systems in pure cultures and consortia. The work had two key objectives: 1) To understand and develop the processes which render biodegradative products innocuous. 2) To combine the fundamental detoxification studies with precise toxicity measurements and statistical/mathematical analyses of metabolic processes to identify those genetic and physiological factors which affect the toxicity of the biodegradation mixtures. This has allowed us to propose and test various means of increasing the biodegradative rates and decreasing the toxicity of the biodegradative mixtures for model compounds and microbial systems. Additionally, we have further developed the OPH system with increased substrate range and catalytic rates againsst complex OP neurotoxins, and further defined the enzymatic activities important in the biotransformation of nitroaromatics.

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FINAL PROGRESS REPORT

Understanding the Factors Controlling Biodegradation of Military Toxic Wastes"

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FOREWORD

This University Research Initiative was an extremely productive and progressive project involving the collaboration of environmental engineers, toxicologists, microbiologists, and biochemists in an integrated study of the factors that affect the destruction and decontamination of the environment by military waste materials. This program provided a particularly robust environment for students who became broadly aware of the interfaces that cross the diverse interests of molecular biology and bioengineering as applied to environmental management and bioremediation of damaged or threatened chemical materials - both military and civilian in nature .

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Statement of the problem studied

This study evaluated three model systems for bioremediation of CW Agents: a) organophosphorus (OP) degradation with the OPH enzyme and related enzymes; b) microbial degradation of nitroaromatics (NAH) and OPs with genetic enhancement; and c) microbial degradation of NAHs and OPs without the OPH augmentation. The primary objectives of the study were:

1) Development of enzyme-based biocatalysts and use them in microbial systems
2) Identify the principal genetic and environmental factors which control the toxicity of biodegradation products and mixtures.
3) Define and model the genetic and environmental factors which control biodegradation of compounds relevant to military toxic wastes.
4) Manipulate the bioreaction systems in order to: a) enhance biodegradation rates of these compounds; b) minimize the toxicity of the biodegradation mixtures; and c) expand the degradative capabilities of the microbial consortia to include mustard-degrading species

Summary of the most important results

1. Modification of Catalytic Capabilities of Organophosphate Hydrolase for the Detection, Destruction, and Decontamination of CW Neurotoxins.
Our studies with the plasmid-borne bacterial gene and its unique broad-spectrum enzyme capable of detoxifying a wide range of organophosphorus neurotoxins ranging from insecticides such as parathion and coumaphos to a chemical warfare neurotoxin) have continued to be directed toward the understanding of how the enzyme can be modified to expand its substrate specificities. The cloned genes have been expressed in a number of biological systems from bacteria to insect tissue culture to native soil fungi, and the enzyme has been purified and characterized from several different sources. Site-directed mutagenesis has been utilized to define the nature of amino-terminal signal sequence and the metallic binding sites. The understanding of the structure of the enzyme is currently being used to rationally redesign the enzyme to manipulate the substrate specificities of the enzyme. The enzyme is being used in developing biosensor technologies for the biological detection of neurotoxins in the environment, biomedical treatments for people who have been exposed to neurotoxins, and stable enzyme preparations for detoxification of neurotoxic contamination through engineering processes.

2. Development of Cell-based Systems for the Complete Destruction of G-agents and Mustards.
Collaborative studies with individual strains and microbial consortia capable of bioconversion of the hydrolysis products of Soman and Sarin degradation have demonstrated the ability of active-growth bioreactors and cryoimmobilized cell systems to degrade these materials. This destruction of the products of the chemical neutralization chemical warfare agents provides the fundamental knowledge necessary to complete the second phase of the Chemical Warfare Convention which requires the non-reversable conversion of CW agents to benign, non-classified chemicals. Integrated chemical-biological systems for the complete destruction of G-agents and Mustards have been designed and tested with surrogates of the agents themselves and the actual product of chemical neutralization.

3. The Aerobic Degradation of Organonitrate Explosives: TNT, RDX, and RMX.
A third area of ongoing research activity involves the demonstration and characterization of the aerobic degradation of organonitrate explosives such as TNT in both mixed and pure culture bioreactors. It is apparent that there are a variety of different metabolic directions for the degradative processes which begin with the reductive formation of mono- and di-amino groups and/or the direct denitration of the carbon ring. Some of these pathways lead to toxic, end-point azoxy-dimers while other pathways appear to lead to denitro reductions, ultimately resulting in ring cleavage and complete destruction of the explosives.

AMPLIFICATION OF SELECTED STUDIES

1. Studies with Organophosphate Hydrolases

Many general neurotoxic insecticides and chemical warfare (CW) agents are members of a class of organophosphorus supertoxicants which exert their effects by binding to acetylcholinesterase (AChE), thus preventing the recycling of acetylcholine (ACh). Organophosphorus hydrolase (OPH; OP anhydrolase, EC 8.1.3.1) is a bacterial metalloenzyme with very broad substrate specificity that can hydrolyze the P-O bond of some organophosphorus neurotoxins at extremely high rates (k_{cat} = >4,000 s^{-1}) which are diffusion limited. In addition, the enzyme can hydrolyze P-F bonds at dramatically different rates from k_{cat} values from 1 to 600 s^{-1} ; P-S bonds with k_{cat} values from 0.02 to 5; and the P-CN bond of Tabun. Although various organisms from bacteria to man have organophosphorus anhydrolase activities, most of the enzymes have limited substrate specificities. (For example they may hydrolyze phosphotriester bonds (P-O) but not P-F or P-CN bonds, and visa versa.) OPH is unique among these OP-degrading enzymes in that it is capable of hydrolyzing both phosphotriesters (including as paraoxon and coumaphos), phosphothioates (such as acephate, Malathion, and VX) and phosphonofluoridates (such as Sarin and Soman, stockpiled by various countries. There can be significant differences within each class (i.e. the hydrolysis of the P-F bonds in chemical warfare agents vary from a k_{cat} Sarin = 350 to k_{cat} Soman = 1.0). As a result of these unusually broad enzymatic characteristics, OPH has received considerable attention as a bioremediation agent with proposed uses including the remediation of CW agent stockpiles, treatment of soil and ground water contaminated with OP insecticides, prophylactic clinical treatment of OP-intoxicated individuals, and for the detection of various OP neurotoxins (Rainina et al., 1995; Simonian et al., 1997: 1999). This gene has been cloned and the three-dimensional structure of the apoenzyme and some bimetallic forms have been evaluated (Benning *et al.*, 1994; Benning *et al.*, 1995; Vanhooke *et al.*, 1996). Organophosphorus hydrolase (OPH) is a bacterial metalloenzyme with broad substrate specificity which was originally isolated from Pseudomonas diminuta and Flavobacterium sp. It is responsible for the initial cleavage step in the degradation of many organophosphate (OP) compounds used as insecticides and chemical warfare agents (CWAs) and has varying degrees of efficacy against different members of this broad class of compounds. OPH has an estimated monomeric weight of 36,000 Daltons which has the overall structure of an eight-stranded α/β barrel as a homodimer. Previous investigations to identify amino acyl residues responsible for binding divalent metal cations coincidentally identified residues which also influences the catalytic activity (k_{cat}) and catalytic specificity (k_{cat}/Km) for various substrates (Lai et al., 1994). For example, the histidinyl residue at position 254, when changed to an argininyl or serinyl residue, improved the catalytic activity and specificity of OPH thirty- to forty-fold against both the chemical warfare agent analog (demeton-S) for VX. Similarly, the histidinyl residue at position 257, when changed to a leucinyl residue, improved the catalytic activity and specificity of OPH more than 20-fold against the chemical warfare agent Soman and its analog NPPMP (Lai, 1994; diSiouti et al. 1998; 1999). Subsequent crystallization of wild-type enzyme and release of crystallographic coordinates of the apoenzyme stimulated interest in the physical basis of these observed improvements. A model was built to incorporate two metal ions into the active site of the wild-type apoenzyme structure, and the model was refined by energy minimization (Wild et al., 1996; Kuhlmann et., 1997). Additional models (H254R, H254S, and H257L) were made in which one of the two

metals at each active site was removed to reflect the experimentally determined metal contents of these altered enzymes. Docking experiments with different organophosphate substrates led to hypotheses regarding the contributions of specific structural moieties to the observed catalytic characteristics of the enzymes. The kinetic and modeling data provided the impetus to design the double mutant H254R/H257L. This enzyme shows greater than 1000-fold improvement of both catalytic activity and specificity for the analogue of chemical warfare agent VX, demeton-S (B.d. Kuhlmann, et al. 1998a,b; 1999a,b). The tremendous improvement in catalytic efficiency suggests that the redesigned OPH may provide a viable alternative technology for the destruction of VX in both the United States and former USSR (Kolalowsti et al., 1997; Rastogi et al., 1998)

The practical applications for bioremediation would require enzymes with greater substrate affinity and enhanced structural stability , as well as improved catalytic efficiency. Our research is involved in the design of new enzymes that will allow the utilization of OPH in remediation technology. These must be tailored for a particular environmental situation, such as the presence of low levels of OPs (enzymes with greater substrate affinity) or for longer term use under harsh environmental conditions (enzymes with greater conformational stability). Rational enzyme design for enhanced biodegradation of OPs will involve information from studies of wild-type OPH and enzymes modified through site-directed mutagenesis. A combination of computer modeling of the enzyme and selected substrates, kinetic characterizations, structural stability studies, and metal content analysis are being used to characterize modified OPH enzymes (Lai et al, 1994: 1997; Grimsley et al., 1997; diSioudi et al. 1999).

Significant improvement of the activity and substrate specificity of this flexible enzyme has been achieved through site-directed mutagenesis of histidinyl residues affecting the metal content and modifying the boundaries of the active site. These results demonstrated that only a single metal is required for enzymatic function and suggested that the changes in catalytic metal geometries are associated with the alteration of substrate specificities. In addition, the combination of substitutions has led to a dramatic increase in catalytic specificities for VX-analogues. These changes are just being analyzed for their direct effects on V-agents; however, the enzyme has been shown to be active against VX (Hoskin et al, 1996; Kolakowski et al., 1997) and Russian VX (Rastogi et al., 1997) .

Site-directed mutagenesis followed by the structural modeling interpretation of organophosphorus hydrolase has permitted with reconstruction of the active site of the enzyme resulting in a 500-fold enhancement of kcat with demeton-S, a structural analogue of the chemical warfare agent for VX. At the same time, there is a slight increase in binding association to the enzyme since the Km value increases from 1.7mM to 920 μM. These changes are accomplished without an overall restructuring of the active site since other substrates show only modest changes in their kinetic activities. The turnover values of these new enzymes are adequate for the development of enzyme-based bioreactors for the destruction of VX and other V-type agents.

X-ray crystallographic coordinates of the wild-type apoenzyme (*H. Holden, U. Wisconsin*) have allowed us to model important features at the active site in collaboration with Dr. Leonardo Scapozza in Zurich (diSioudi, et al, 1998: 1999). As a result, critical characteristics have been identified that allows rational design of OPH for bioremediation. Some proposed changes involve modification of polarity in or around the active site pocket in order to accommodate substrates for which the wild-type enzyme has low catalytic efficiency or specificity. Other changes may add bulk to the active site in order to improve the positioning of some substrates. In some cases, modification

will hopefully improve substrate fit through reduction of steric hindrance. The capacity for further improvement is quite remarkable, and the opportunity for a variety of biotechnical applications is available.

The use of OPH in the remediation of hazardous compounds is still compromised by our limited understanding of its stability and how the enzyme would behave in environments most typical of where it would be used. Since OPH is insoluble in organic solvents which are suitable for solubilization of nerve agents, enzyme-based detoxification of bulk nerve agents (for instance in an existing war head or a concentrated spill of pesticide) must often rely on the use of OPH in biphasic mixtures. This fact points to the first challenge: In order to efficiently function in detoxification of bulk nerve agents OPH must be stable in the presence of organic solvents. In order to increase the productivity of the process we must immobilize the enzyme in the most effective way currently available. Therefore, another challenge is preeminent: OPH must have the highest possible stability under adverse conditions. For these and related reasons, we have begun to study the folding of OPH to determine its conformational stability. So far, we have determined that under our laboratory conditions, OPH is a very stable protein with a free energy change of unfolding equal to approximately 40 *kcal/mol*, the highest reported so far for any oligomeric or single domain protein. (Grimsley *et al.*, 1997). Now that we have established the necessary conditions to monitor OPH stability, we are now in a position to evaluate the stabilization factors that are required for remediation technology under a variety of environmental conditions.

Studies with Biodegradation of Aromatic Explosives

2,4,6-trinitrotoluene (TNT) is a nitroaromatic compound commonly used in munitions such as shells, bombs, and grenades, as well as industrial uses. It is often the main contaminant of soil and ground water at its manufacturing, processing, and disposal facilities. The environmental impact at production sites is compounded by the requirement of large amounts of water in the production of munitions. In the past, TNT contaminated wastewater has been discharged either on the ground or in shallow lagoons. TNT concentrations at these sites can be as high as 100 mg liter $^{-1}$ (440 μM) in water and 12,000 mg kg^{-1} in soil. With production levels of over 1,000 tons per year in the 1980s and the high number of contaminated military sites, TNT disposal and remediation has become a significant environmental concern. The high toxicity and mutagenicity of TNT and some of its metabolites, as well as its recalcitrant characteristics, has led to considerable interest in its fate in the environment. Numerous cases of munitions workers which have developed liver damage and anemia due to TNT exposure have been documented. In addition, TNT has been shown to have toxic effects in rats and, fish, algae and oyster larvae. In addition, TNT has been shown to be a mutagen. Consequently, the remediation of contaminated sites and the safe disposal of military stockpiles has become increasingly important.

The traditional treatment of TNT waste includes physical and chemical treatments such as wet air oxidation, thermal decomposition, chemical reduction, photocatalytic degradation, air stripping, filtration, fixation, incineration, *in situ* vitrification, and solvent extraction. The most commonly used of these techniques are fixation, vitrification and incineration. All three of these methods generate toxic by-products which necessitate disposal in hazardous chemical landfills. This, plus the high cost

of these treatments, has resulted in a renewed emphasis on the development of alternative technologies such as bioremediation. Most of the research into the biological remediation of TNT has involved anaerobic bacteria and fungi, and the efficiacy of using four different strains of white rot Fungi in liquid culture has been shown in our studies (Donnelley et al, 1997). Of the biological systems investigated, fungi have proven to be the most effective for bioremediation of TNT, although there is an important limitation in that the hydroxylamino intermediates produced are toxic to the organism. As a result, the rates of degradation are limited by the accumulation of these metabolites. Several researchers have evaluated composting TNT; however, this increases the amount of waste material and the end products have been poorly characterized. Studies exploring the capabilities of aerobic bacteria have only recently begun to appear in the literature. A unique thermodynamic capability study done by M. Shelley (Shelley et al., 1996) has modeled the varying pathways which might be potentially involved in the degradation of TNT (Collie et al., 1995), and the in situ degradation of RDX and HMX as well as TNT has been demonstration in studies in our laboratories (Hallgarth et al., 1997; Kalafut, 1998).

The biochemical transformation of TNT has proven to be complex, and many potential metabolites are more toxic than the parent compound. The toxicity and extreme recalcitrance of TNT is a direct result of its chemical structure. TNT is a tri-substituted toluene, with three nitro ($-NO_2$) groups on the aromatic ring. The reduction of the nitro group results in the formation of reactive intermediates which are responsible for the majority of the toxic effects. The nitro group is first reduced to a nitroso group by the addition of two electrons. This can occur by either a sequential addition of single electrons, or a single step addition of two electrons.

The sequential addition of single electrons results in the formation of an oxygen radical which will enter into a futile redox cycle in the presence of molecular oxygen. The nitro radical is oxidized back to the initial nitro form with the formation of a superoxide. Because of this oxygen interaction, the single electron pathway is characterized as oxygen sensitive. The single step addition of two electrons forms the nitroso intermediate directly and requires a reduced pyridine nucleotide cofactor such as NADPH or NADH to donate the electrons. This mechanism results in the oxidation of the cofactor, which can be monitored in a spectrophotometric assay.

Once the nitroso intermediate is formed, the sequential addition of two electron pairs results in the formation of the hydroxylamino intermediate, and finally the terminal reduction product, which is an amino ($-NH_2$). The sequential reduction of the nitro groups requires an increase in the reducing potential of the environment. The reduction of the first nitro group to the amino requires a redox potential of -10 mV, which increases to -140 mV and -290 mV for the second and third groups, respectively. The strongly reducing conditions necessary to reduce TNT to TAT have only been documented for anaerobic systems with a redox potential of -200 mV or stronger.

The high degree of substitution of the aromatic ring in TNT contributes to the recalcitrance of the compound in the environment. The mineralization of lesser-substituted aromatic compounds such as 24DNT, 2NT, 4-nitrophenol and nitrobenzene has been well described. In order for ring cleavage to occur, oxygenases must first hydroxylate the ring to form catechols. This requires adjacent unsubstituted carbons, which are not present in TNT. Both mono- and dioxygenases can use aromatic compounds as substrates to form mono- and dihydroxy compounds. A second set of oxygenases can then break the ring through either an *ortho* or *meta* pathway, with the ultimate transformation products being simple three or four carbon compounds that can be used for energy

production. In order for the mineralization of TNT to occur to any degree of efficiency, one or more groups on the ring need to be removed.

These studies have led to the characterization of several novel aerobic strains of bacteria that biotransform TNT in different ways (Kalafut et al., 1998). The strains were all isolated from TNT contaminated sites and include a *Pseudomonas aeruginosa* isolated by R. P. Naumova, and a *Bacillus* sp. and *Staphylococcus* sp. isolated from the Savannah Army Depot, IL by Dr. Kirby C. Donnelly (Texas A&M University). They differ in their toxic sensitivity to TNT, rate of TNT metabolism, growth rates in the presence of TNT, and the way they partition TNT in the biomass. Three purified enzyme fractions were isolated from the *Bacillus* sp., with two very distinct sets of characteristics for substrate and cofactor preferences, as well as kinetic parameters. None of the aerobic bacteria strains evaluated in this study can use TNT as a sole carbon or nitrogen source. However, all three of the strains can co-metabolize TNT if the cell density is sufficiently high. The *Bacillus* and *Staphylococcus* strains have a very interesting characteristic in that they require TNT to grow in MM, and the *Staphylococcus* sp. also required TNT in LB. The *Bacillus* sp. and the *Staphylococcus* sp. both produced 2A4NT in MM, which has adjacent unsubstituted carbon atoms.

The *Bacillus* sp. contains at least two unique enzymatic activities Kalafut, Ph.D. Dissertation, 1998). These activities are separated by ion exchange chromatography, illustrating differing physical characteristics. Within the two distinct activities, $\alpha\beta55$ and $\gamma36$ show strong preferences for the *para* position of the ring and NADPH as the cofactor, while the second enzyme can utilize the *ortho* position and NADH as well. They differ kinetically, with $\alpha\beta55$ and $\gamma36$ exhibiting Michaelis-Menten kinetics, while Peak 2 exhibits sigmoidal saturation curves, indicating multiple active sites with a cooperative response to substrates. The probing of *Bacillus* sp. genomic DNA with a degenerate probe from $\alpha\beta55$ and subsequent seqeuncing reveals 73% homology to a specific area of the *Bacillus subtilis* genome. The first 34 amino acids of Enzyme A correspond to the start of the *yhaR* ORF.

The endpoint for bioremediation is somewhat ambiguous. The loss of toxicity, removal of the parent compound, or the lack of bioavailability have all been used to describe the remediation of a compound. However, the mineralization of a xenobiotic is unequivocal evidence for the successful degradation of the compound of interest. The mineralization of nitroaromatic compounds hinges on the ring being available to oxygenase enzymes for the formation of catechols, and the subsequent breakage of the ring.

The addition of reducing agents to the enzyme assay opens up the possibility of a new avenue for the complete degradation of TNT. This is believed to be the first example of the transformation of TNT to 2NT and 24DNT in an enzymatic reaction. As discussed above, this would allow unprecedented access to the ring by oxygenases, which is necessary for ring cleavage and mineralization.

3. Bioreactor Design and Bioremediation Usages

It has been shown that it is possible to use immobilized cells for the degradation/detoxification of a variety of different chemical waste or chemical threat agents. One of the most unique aspects of this work has been the cryoimmobilization of a variety of different bacteria - both native and genetically engineered, as pure cultures or part of undefined consortia.

The results of these studies have demonstrated the potential for using cryo-immobilized

non-growing OPH+ cells for bioremediation of CP in cattle dip waste (Kim et al., 1999), for a variety of neurotoxic pesticides and chemical threat agents (Hong et al., 1998) as well as the degradation products of actual chemical warfare agents such as sarin (Zhang et al. 1999) or sulfur mustard (Rainina et al., 1998).

Biodegradation efficiencies of CP in cattle dip waste (Kim et al., 1999; Kim, Ph.D. Dissertation, 1999), thiodigylcol (Rainina et al., 1998), and IPMP (the neutralized sarin product) were significantly enhanced by utilizing cryo-immobilized non-growing cell systems as compared to degradation by either the pure cultures of the native bacteria or by immobilized natural microbial consortium. Such enhanced efficiencies were attributable both to the increased biomass availability in the systematically designed high-cell-density systems and to the ability of the cryogel to exclude inhibitory factors hindering metabolic activity of the various cells from entering the biocatalyst particles. Cryo-immobilized cells retained much of their activity over a four month period of use and storage, indicating good catalytic and storage stability of the cryo-immobilized cells. Long-term incubation of cryo-immobilized cells demonstrated the potential for complete mineralization of various toxic waste materials (such as used CP in cattle dip) by using additional microorganisms such as the natural consortium existing in the spent cattle dip. Cryo-immobilized OPH+ cells were able to enhance the bioavailability of CP for the native microflora and to activate them for further degradation of the immobilized cell product.

These studies have shown that cryo-immobilized non-growing cell systems would provide effective methods for rapid bioconversion processes including decontamination, detoxification and degradation of environmental toxins, including CP, in the commonly-occurring cases where low growth rate and substrate and/or product inhibition are a great concern. These new technologies provide an exciting opportunity for a variety of biotechnology applications in the future.

"Understanding the Factors Controlling Biodegradation of Military Toxic Wastes"

Studies from an ARO University Research Initiative Program

Selected Publications (1994-1999)

James R. Wild
Robin L. Autenrieth
Bruce E. Dale
James S. Bonner
Charles Kenerley
Kirby C. Donnelley

The Texas A&M University System

Texas A&M University
The Texas Engineering Experiment Station
The Texas Agriculture Experiment Station

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"An Enzyme-based Biosensor for the Direct Determination of Diisopropylfluorophosphate." 1999. A.L. Simonian, B.D. diSioudi, and J.R. Wild. Analytic Chimica Acta. 389:189-196.

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